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Plasma concentrations of testosterone and nandrolone in racing and nonracing intact male horses

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Pennsylvania (PA) State Racing Commissions regulate the endogenous androgenic steroid, testosterone (TES), in racing intact males (RIM) by quantification of TES in post-race samples. Post-race plasma samples (2209) collected between March 2008 and November 2010 were analyzed for TES, nandrolone (NAN), and other anabolic steroids (ABS). Of the 2209 plasma samples, 2098 had quantifiable TES ≥25 pg/mL. Plasma (mean ± SD) concentrations of TES and NAN in RIM were 329.2 ± 266.4 and 96.0 ± 67.8 pg/mL, respectively. Only 64.6% of RIM had quantifiable concentration of NAN, and there was no relationship between TES and NAN. Plasma TES concentrations were significantly ($P < 0.0001$) higher during the months of April, May, June, July, and August. A significantly higher ($P < 0.006$) plasma TES was observed in Thoroughbred (TB) (347.6 ± 288.5 pg/mL) vs. that in Standardbred (STB) (315.4 ± 247.7 pg/mL). Plasma concentrations of TES from breeding stallions (BS) were 601.6 ± 356.5 pg/mL. Statistically significant ($P < 0.0001$) lower plasma concentrations of the two steroids were observed in RIM horses. Based on quantile distribution of TES in the RIM and BS populations, 99.5% were at or below 1546.1 and 1778.0 pg/mL, respectively. Based on this population of RIM, the suggested upper threshold plasma concentration of endogenous TES in horses competing in PA should remain at 2000 pg/mL.

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INTRODUCTION

The Pennsylvania State Racing Commissions regulate the use of anabolic (ABS) and androgenic (ANS) steroids in equine athletes by monitoring post competition plasma samples for the presence of these agents. Testosterone is endogenously produced in measurable concentrations in plasma or serum in the intact male horse; therefore, the concentration of this hormone was monitored (Soma *et al.*, 2008). Plasma samples were chosen over urine because the plasma concentration is a more reliable index of the use of drugs and the pharmacological action of many drugs is based on plasma and tissue concentrations instead of urine (Soma *et al.*, 2007a,b).

Androgenic steroids in the intact male horse include androstenedione, dihydrotestosterone, dehydroepiandrosterone, androstanediol, and testosterone, of which testosterone is the dominant steroid (Ganjam *et al.*, 1973). A common method used for the quantification of ANS in plasma has been radioimmunoassay, which is not as specific as the direct measurement by liquid chromatography-mass spectrometry due to cross reactivity with other steroids (Silberzahn *et al.*, 1988).

The purpose of this study was to determine the concentrations of TES and NAN in plasma samples collected from a large population of RIM competing in PA over 3 years of racing seasons, compare plasma concentrations of the male hormones TES and NAN in RIM and BS and suggest an upper tolerance limit for endogenous TES in RIM for regulatory purposes.

MATERIALS AND METHODS

From March 1st 2008 to November 1st 2010, post-race plasma samples were collected from winning RIM (2209) STB and TB horses and analyzed for TES, NAN, and ABS. Samples were collected in vacuum tubes with potassium oxalate (20 mg) and sodium fluoride (25 mg) used as anticoagulant (Kendall,

Mansfield, MA, USA) and all were part of the routine postrace sample collection drug surveillance program. Due to the time spread of the sample collection, the samples from a horse racing more than once were treated as independent samples.

Plasma TES concentrations (≥25 pg/mL) of samples (2098) collected from RIM TB and STB horses were compared with those of 118 serum or plasma samples from 95 normal BS of various light and heavy breeds (Thoroughbred, Standardbred, Warmblood, Arabian, Gypsy Vanner, Quarter Horse, Clydesdale, Saddlebred, Connemara, Welsh and Shetland) collected either on-farm or at our reproduction referral clinic between January 2007 and August 2009; all were free of ABS. Horses aged 2–4 years were considered colts and those aged 5 years and older were horses.

European and Asian racing jurisdictions including the Fédération Équestre Internationale banned the use of androgenic and anabolic steroids in horse competitive events for the past decades. The State of Pennsylvania was the first racing jurisdiction in the United States to use plasma concentrations to ban the use of ABS and ANS in all competing racehorses; the enforcement of the ban went into effect in March 2008. A transition period from March 1st to October 1st, 2008 was put in place to allow horses to compete while the use of these compounds was being eliminated from racehorses. Testing on all horses was conducted to assure that concentrations of ABS in all racehorses and of ANS in mares and geldings were on a steady decline to reflect withdrawal of these compounds from all horses competing in PA. All veterinarians and racing organizations were notified of the proposed enforcement in ABS policy effective January 2008 to allow sufficient time to discontinue the use of these steroids before March 2008.

Quantification of ABS and ANS

Method of quantification of ANS and ABS in plasma was performed using triple-stage quadruple mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) as previously described (Guan *et al.*, 2005; Guan *et al.*, 2006; You *et al.*, 2009). In this study, a more sensitive and high-throughput UPLC-MS/MS method was developed for quantification of eight major ABS and ANS, i.e., trenbolone, boldenone, nandrolone, methandrostenolone, testosterone, mibolerone, stanozolol, and the designer steroid tetrahydrogestrinone (THG) in equine plasma (You *et al.*, 2009) The analytes were recovered by liquid–liquid extraction using methyl *tert*-butyl ether. Analyte separation was achieved on a 1.9 μm column using UPLC system. LC separation was based on mobile phase gradient using 2 mM ammonium formate buffer and MeOH as mobile phase. Analytes were detected using a triple quadrupole mass spectrometer by positive electrospray ionization mode with selected reaction monitoring (SRM). Deuterium-labeled testosterone and stanozolol were used as dual internal standards for quantification. Total analysis time was 5 min.

The method was validated for specificity, matrix effect, linearity, sensitivity, accuracy, precision, and robustness. In brief, analytes with sharp and symmetrical peaks were eluted within 4 min. Specificity study results indicated that no endogenous interferences were observed at the same retention time as of each AAS, suggesting that the method is highly specific. The quantification linear range was 25–10 000 pg/mL. There was no significant matrix effect observed for all analytes in plasma. Intra-day precision and accuracy were 2–15% and 91–107%, respectively. Inter-day precision and accuracy were 1–21% and 94–110%, respectively. To date, the method has been successfully used in the analysis of more than 20 000 postrace samples and is reproducibly reliable. Standard Operating Procedures employed by our laboratory for quantification of the analytes meet requirements for accreditation by the American Association for Laboratory Accreditation and ISO 17025 International Guidelines. Concentrations of TES below LOQ were considered 'detected but not quantified'.

Statistical analysis

One-way and two-way ANOVA were used for data analysis (JMP Version 8.0; SAS Institute Inc., Cary, NC, USA). Plasma concentrations of TES were expressed as mean and standard deviation (SD). Univariate plot was used to determine distribution of the continuous data and to establish the 99.5% quantile. Significance was indicated when $P < 0.05$.

RESULTS

Testosterone concentrations in racing intact males

Postrace plasma samples (2209) from RIM were collected between March 2008 and November 2010 and analyzed for TES, NAN, and other ABS. Of the 2209 plasma samples analyzed, 111 had TES of less than 25 pg/mL, of which 55 had no detectable TES. Plasma samples without or only detectable TES contained either NAN (110.3 ± 186.8 pg/mL), stanozolol (461.6 ± 1029.0 pg/mL), or boldenone (186.8 ± 222.6 pg/mL). These results refer only to plasma samples collected and analyzed during the transition period (March 1–October 1, 2008) when the plasma concentrations of ABS were decreasing in all horses competing in PA because of the withdrawal period in preparation for the enforcement of the ban on ANS and ABS by the Horse and Harness Racing Commissions.

Seasonal variations

In RIM, the plasma concentrations of TES started to increase in March and decreased in September (Fig. 1). Plasma concentrations of TES in RIM were significantly ($P < 0.0001$) greater during the months of April, May, June, July, and August than in other months; the mean response was 329.2 ± 266.4 pg/mL.

Differences in the breed of the horse

In this population of RIM, 942 plasma samples were from TB and 1267 from STB. A significantly higher ($P < 0.006$) plasma TES

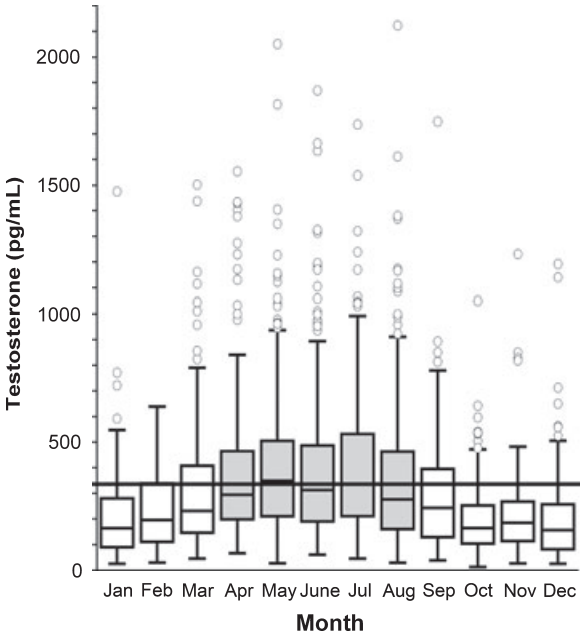

Fig. 1. Box plot of the ranges in plasma concentrations of TES in racing intact male (RIM) horses from March 2008 to November 2010 from both Thoroughbred (TB) and Standardbred (STB) racetracks in PA. Grey boxes were significantly different ($P < 0.0001$). Solid line is the mean response of the data. Each box encloses 50% of the data with the median value displayed as a solid line in the box. Upper quartile and lower quartile of each box mark the 25% and 75% quartiles of the population. Extended line from the top of each box represents the inter-quartile range times 1.5 plus the upper quartile. Individual points (open circles) beyond the extended lines are outliers. The lower extended line is set by the limit of quantification, 25 pg/mL ($n = 2098$).

concentration was observed in TB (347.6 ± 288.5 pg/mL) vs. that in STB (315.4 ± 247.7 pg/mL). Only 64.6% of the RIM with TES plasma concentrations >25 pg/mL had quantifiable concentrations of NAN. Plasma concentrations of NAN were significantly higher ($P < 0.0001$) in TB (106.9 ± 76.4 pg/mL) than in STB (87.7 ± 59.3 pg/mL). There was no correlation between TES and NAN plasma concentrations in either of the breeds.

Age differences

Significant differences ($P < 0.0001$) were observed in plasma concentrations of TES between TB colts and horses, but not in STB colts and horses. Plasma concentrations of TES in TB colts and TB horses were 392.2 ± 310.1 and 240.2 ± 188.4 pg/mL, respectively, whereas those of STB colts and horses were 312.1 ± 220.8 and 327.7 ± 294.1 pg/mL, respectively. Statistically significant age-related differences were noted at two of the six racetracks associated with the population of horses competing at these racetracks (Table 1). Significant differences were not observed in plasma concentrations of NAN between colts and horses.

Differences due to racetrack

Significantly ($P < 0.001$) lower plasma TES concentrations were observed at one STB racetrack (MD). Statistically significant age-related differences were noted at two of the six racetracks associated with the population of horses competing at these racetracks (Table 1). One TB racetrack (PID) had significantly ($P < 0.001$) higher plasma concentrations of NAN (144.0 ± 93.7 pg/mL) compared with other racetracks (91.8 ± 63.4 pg/mL).

Table 1. Plasma concentrations of testosterone from post-race samples collected from racing intact male Standardbred (STB) and Thoroughbred (TB) racehorses competing at Pennsylvania racetracks ($n = 2098$ samples)

Racetrack	Age of horses	Number of horses	Testosterone (pg/mL)
CD (STB)	4.6 ± 2.0*	432	333.5 ± 269.6
FAIR (STB)	3.0 ± 1.6*	75	378.7 ± 247.9
MD (STB)	4.1 ± 1.8	443	274.2 ± 201.7†
PD (STB)	4.1 ± 2.0	248	338.3 ± 271.9
PID (TB)	3.8 ± 1.6	137	344.7 ± 223.6
PN (TB)	4.2 ± 1.5	245	348.4 ± 282.0
PP (TB)	3.9 ± 1.5	518	347.9 ± 306.6

*Significantly different ($P < 0.001$) in age from one another and from the other five racetracks. †Significantly different ($P < 0.0003$) in TES from all other six racetracks.

Comparison of racing (RIM) and nonracing male (BS) horses

Mean plasma concentrations of TES in RIM and BS were 332.0 ± 267.8 and 601.6 ± 356.5 pg/mL, respectively. Concentrations of TES in RIM were statistically lower ($P < 0.0001$) than in BS.

Distribution plot of TES in 2098 plasma samples collected from RIM between March 2008 and November 2010 showed a median value of 254.5 pg/mL, the 25% and 75% quantiles were 151.0 and 424.5 pg/mL, respectively. Based on quantile distribution of this population, 99.5% was at or below 1546.1 pg/mL and only 10 horses were above that value. The highest TES plasma concentration in RIM horses was 2123.0 pg/mL (Fig. 2).

A distribution plot of TES collected from 118 BS showed a median value of 557.5 pg/mL, the 25% and 75% quantiles were 319.3 and 785.5 pg/mL, respectively. Based on quantile distribution of this population, 99.5% of the samples were at or below 1778.0 pg/mL (Fig. 2).

DISCUSSION

Prior to March 2008, the normal plasma concentration of TES in RIM in PA was difficult to establish due to the widespread use of ABS and TES in the racehorse population. The existing ban on the use of ANS in mares and geldings and ABS in all horses was enforced effective from March 2008 by the PA Racing Commissions. Testing in all horses was conducted to assure that concentrations of ABS in all horses and ANS in females and geldings were on a steady decline during the transition period. Veterinarians and racing organizations were notified of the

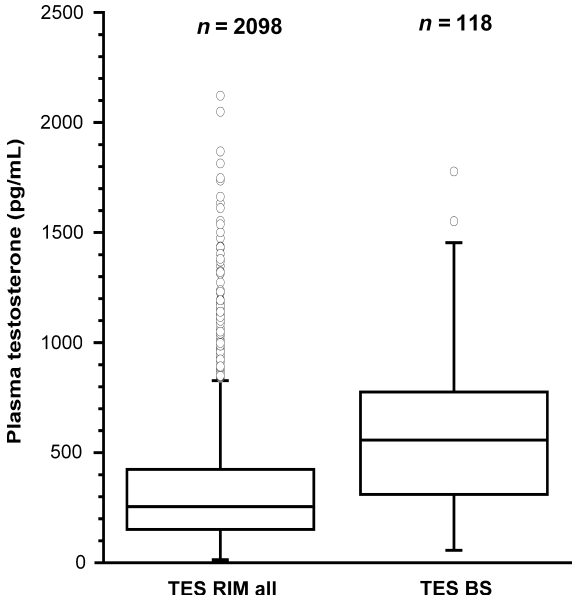

Fig. 2. Box plot of the range in plasma concentrations of TES in racing intact males (RIM) and breeding stallions (BS). Each box encloses 50% of the data with the median value displayed as a solid line in the box. Median plasma concentrations were 254.5 and 557.5 pg/mL for RIM and BS, respectively. Upper quartile and lower quartile of each box mark the 25% and 75% quartiles of the population. Extended line from the top of each box represents the inter-quartile range times 1.5 plus the upper quartile. Individual points (open circles) beyond the extended lines are outliers. The lower extended line is set by the limit of quantification, 25 pg/mL.

impending enforcement of the ban on the use of ABS and ANS in January 2008. Beginning in March 2008, measurable plasma concentration of ABS in all horses including RIM drastically dropped compared with a previous report (Soma *et al.*, 2007a,b).

The effects of ABS on endogenous TES were noted during the 8-month transition period (March 1–October 1, 2008) in which 8.3% of the racing males had no quantifiable or plasma TES lower than the LOQ of 25 pg/mL. During the 10-month period and subsequent to this period, the number of horses with TES concentration lower than 25 pg/mL declined as the ABS positives virtually disappeared. In the regulation of ABS and ANS, once sufficient time is allowed for withdrawal from these agents, it is unlikely for a horse to be administered these agents again due to the lengthy withdrawal time.

In the RIM horses with low or no detectable plasma concentrations of TES, the administration of ABS in the months before the start of this ban probably contributed to suppression of the production of endogenous TES. Results of numerous studies have shown the negative effect of the administration of ABS on stallion sperm count and motility, testicular size and scrotal width (Squires *et al.*, 1982; Blanchard, 1985; Garcia *et al.*, 1987). Following the cessation of administration of ABS or TES, recovery from the effects of administered ABS or TES occurs in about 90 days (Squires *et al.*, 1981). Studies in men have also shown a reduction in plasma TES following the administration of ABS (Harkness *et al.*, 1975; Kilshaw *et al.*, 1975; Holma & Adlercreutz, 1976; Clerico *et al.*, 1981).

Plasma concentrations of TES and NAN were lower in RIM than in BS, which confirms results from previous studies (Soma *et al.*, 2008). Concentrations of TES ranging from 65 to 1600 pg/mL have also been reported (Cox *et al.*, 1973). Studies of harem and bachelor stallions, as well as breeding and non-breeding mature non-racing stallions, indicate a wide range of testosterone concentrations among individuals and throughout the year, with a general pattern of greater testicular size and testosterone concentrations when stallions are exposed to females and breeding (McDonnell & Murray, 1995). Variation due to exposure to breeding and mares, no doubt, contributed to the higher concentrations of TES in BS compared with RIM.

Studies in human male athletes have shown that concentrations of TES in plasma were lower in long distance runners, master class runners, elite amateur cyclists, and wrestlers (Duclos *et al.*, 1996; Slowinska-Lisowska & Majda, 2002; Hackney *et al.*, 2003; Kilic *et al.*, 2006) than in non-athletes. Based on the racing schedules of horses competing year-round, a similar effect of training with the stress of competition may also contribute to the lower plasma concentrations of TES in RIM.

Standardbred racehorses had lower concentrations of TES than TB; this observation may be due to extensive training and more frequent competition than TB. Standardbred training is more of an endurance type of training and STB horses on the average race more frequently than TB horses. The lowest plasma concentrations of TES were observed at a STB racetrack (Table 1) that competes year-round and horses generally race every 7–10 days suggesting that extensive training and frequent competition can depress TES concentration in horses as in human athletes.

All racetrack samples were collected postrace and collection time varied from 30 to 90 min after competition. It is not clear what influence acute exercise had on the concentration of TES in RIM. Under resting conditions, the plasma concentration of TES in RIM might be even lower than post race.

Studies in human male athletes have shown an increase in many hormones including TES following strenuous exercise (Jensen *et al.*, 1991; Hackney *et al.*, 1995; McMurray *et al.*, 1995), which decreased toward baseline concentration within 2 h. Some investigators have suggested that the apparent increase in TES was equal in magnitude to and accounted for by exercise-induced alteration of plasma volume, resulting in no change in total plasma TES concentration (Wilkerson *et al.*, 1980; Kraemer *et al.*, 1992).

The natural breeding season for the equine is from May to July (Johnson *et al.*, 1991). Seasonal variations in basal plasma TES concentrations have been reported in non-racing stallions with a low of 200 ± 100 pg/mL in January to a high of 1400 ± 300 pg/mL in April (Kirkpatrick *et al.*, 1977; Aurich *et al.*, 2003). Using liquid chromatography-mass spectrometry for analysis, seasonal variation was also observed in RIM and BS (Soma *et al.*, 2007a,b). Testosterone concentrations in these groups showed seasonal variations. Diurnal variation in total androgens has also been noted in normal stallions, from highest at 06:00 in the morning to lowest at 18:00 in the evening (Ganjam & Kenney, 1975).

Stallions can convert TES to estrogens (estrone and 17α-estradiol) by the Leydig cell of the testis. Neutral C18-steroids such as 19-nortestosterone (nandrolone) and 19-norandrostendione have also been measured. The urinary excretion of NAN in the intact male and other 19-neutral steroids have been demonstrated (Bedrak & Samuels, 1969; Houghton *et al.*, 1984; Dintinger *et al.*, 1989; Dumasia *et al.*, 1989). Results of subsequent studies using radioimmunoassay for quantification of NAN supported the endogenous secretion by its presence in both plasma and testis. The assumption made by the authors was that at the time of collection of the plasma sample, the biosynthesis of NAN from TES was not taking place in all horses, as TES was quantified in all the male horses involved, but NAN was not (Benoit *et al.*, 1985). Results of our present study support observations that NAN was not detected in all RIM and when present, concentrations of NAN were not related to those of TES. Nandrolone has been quantified in the plasma of BS, but not in non-racing geldings and females (Soma *et al.*, 2007a,b).

Absence of NAN in approximately 40% of plasma samples collected from racing and nonracing male horses suggests that the production of NAN was a post-collection process rather than endogenous production in the horse. We are suggesting that the presence of NAN resulted from spontaneous *ex-vivo* conversion from a precursor in plasma samples that was not immediately analyzed following collection (You *et al.*, 2010). This aspect will be discussed in detail elsewhere. Post-race blood samples used in this study vary in time from collection to final processing as they may be retained at the racetrack for up to 2–4 days prior to shipment to the laboratory. Samples were refrigerated at the racetrack, but not during transportation to the laboratory. Delivery of test samples to the laboratory was by next day service.

Other studies have suggested that the detection of NAN in the extract of the testis and urine could be an artifact of the analytical procedure (Dumasia *et al.*, 1989; Houghton *et al.*, 2007). In drug surveillance programs, the separation of apparently naturally occurring NAN from administered NAN in the urine of intact male horses was based on the urine ratio of quantified 5α-estrane-3β,17α-diol and 5(10)-esrtane-3β,17α-diol or the total quantity of 5(10)-estrane-3β,17α-diol (Dehennin *et al.*, 2007).

Urinary NAN may be a product of decarboxylation of testosterone-19-oic acid and NAN detected may be an artifact (Houghton *et al.*, 2007). We have suggested that LC-MS method of analysis was unlikely to produce decarboxylation in plasma due to the absence of acid solvolysis of the sample extracts and the more gentle procedure used for the extraction of steroids from plasma (Guan *et al.*, 2005; You *et al.*, 2009).

Boldenone sulphate was reported to be an endogenous hormone in the male horse because it was detected in urine samples collected from intact male horses (Ho *et al.*, 2004). However, we have not detected boldenone in any of the plasma samples collected from BS and RIM horses (Guan *et al.*, 2005; Soma *et al.*, 2008). Following full implementation on the ban of the use of ABS and ANS in PA, boldenone was not detected in plasma samples collected from RIM horses. This result suggests that boldenone is not endogenous in equine plasma.

In Monitoring test samples for the presence of ABS, ANS, and other drugs, plasma is the matrix of choice as concentrations in urine do not provide a reliable index of the pharmacological actions of drugs. Urine is an unreliable medium to establish withdrawal times for drugs administered. The sensitivity of current analytical systems allows the use of plasma or serum and does not require the concentrating effects of urine for the detection of low concentrations of drugs administered to competing horses. Racing jurisdiction that uses urine to regulate the administration of ABS and ANS does not regulate TES in the intact male. This is due to the complex mixture of the various metabolic products of the male hormones. Detection, quantification and confirmation of the presence of anabolic steroids are easier and more reliable in plasma or serum than in urine. Anabolic steroids are extensively metabolized in the body, and the metabolites are excreted into urine, which further complicates the analytical processes and the results obtained. Consequently, parent ABS or ANS may not be detectable in urine, and the metabolites are difficult to confirm due to interferences from endogenous androgenic steroids that are similar in chemical structure to the metabolites of exogenous anabolic steroids administered. Plasma or serum is a cleaner matrix and the extraction of drugs from this matrix is simplified, faster and allows the use of rapid through-put analytical procedures. A very low concentration in urine may not reflect a measurable concentration of drug in blood. Hair is not commonly used as a matrix in the racing industry because of logistics in the collection, and in some states, it would require a legislative mandate to include hair as one of the matrices for drug testing. In addition, unlike blood and urine, hair is used in retrospective testing to estimate the length of time the drug has been used in the horse or human athlete. As ABS in all horses and ANS in geldings and females have zero tolerance, length of time of abuse of these drugs in the horse is irrelevant.

Based on the current data, the upper threshold plasma concentration of TES in RIM should remain at the previously suggested plasma concentration of 2000 pg/mL (Soma *et al.*, 2008).

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